

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

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neuromodulatory mechanisms of the pedunculopontine nucleus**

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**INVESTIGATION OF DIRECT NEURONAL AND INDIRECT, ASTROCYTE-DEPENDENT
NEUROMODULATORY MECHANISMS OF THE PEDUNCULOPONTINE NUCLEUS**

By Csilla Bordás, Molecular biology MSc degree

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Head of the **Examination Committee:** János Szöllősi PhD, DSc
Members of the Examination Committee: Klára Matesz, MD, PhD, DSc
Rita Gálosi MD, PhD

The Examination takes place at 2.306 office at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, at 10:30 am, 25th October, 2017.

Head of the **Defense Committee:** János Szöllősi PhD, DSc
Reviewers: Attila Tóth PhD, DSc
Imre Kalló MD, PhD

Members of the Defense Committee: Klára Matesz MD, PhD, DSc
Rita Gálosi MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1 pm, 25th October, 2017

INTRODUCTION

The pedunculopontine nucleus (PPN) is a cholinergic structure in the tegmentum of the midbrain. The nucleus is located laterally from the superior cerebellar peduncle, and medially from the lateral lemniscus. It is bordered by the substantia nigra rostrally, and by the cuneiform and subcuneiform nucleus dorsally. This is one of the main cholinergic cell groups (besides the laterodorsal tegmental nucleus). PPN is not a homogenous structure, as it is composed of neurons with different neurochemical markers, and they also differ in the size of their soma and dendritic tree. The pedunculopontine nucleus is a part of the reticular activating system; its best known role is the regulation of sleep, wakefulness and motion.

The reticular activation system (RAS) is known as a structure which plays an important role in the organization of arousal and attention. The RAS is a diffuse network of nuclei that gives rise for ascending fibers to the cortex while receiving inputs from sensory structures. Several brainstem nuclei are also part of this network with PPN, such as laterodorsal tegmental nucleus, locus coeruleus, parabrachial nucleus, raphe nuclei, ventral tegmental area; and functionally include certain areas of the hypothalamus and the cholinergic basal forebrain nuclei. Inputs are generated by pain, touch,

heat, visual and acoustic stimuli. It reaches the cerebral cortex through two main pathways, which are dorsal (via the thalamus) and ventral (via the basal forebrain and hypothalamus).

Besides the PPN, there are other cholinergic structures in the brain. The non-motor cholinergic nuclei are located in the basal forebrain and in the rostral part of the brainstem, projecting to other brain areas. These brainstem and forebrain cholinergic structures are marked as Ch1-Ch6 regions. Ch1 and Ch2 belong to the medial septal nucleus and the nucleus of the vertical diagonal band, which form the most important cholinergic innervation areas of the hippocampus. Cholinergic nuclei of the septum form the Ch1 region. The pathway from the nucleus of the vertical diagonal band forms the Ch2 region, which is responsible for the cholinergic innervation of the hippocampus. The Ch3 region belongs to the lateral part of the nucleus of horizontal diagonal band, which is the main cholinergic input of bulbus olfactorius. The Ch4 pathway provides cholinergic innervation for the cortex and amygdala, originating from the magnocellular cholinergic nuclei of the nucleus basalis. Ch5 is the pedunculopontine nucleus, which is located in the pontomesencephalic reticular formation, and Ch6 is the laterodorsal tegmental nucleus (LDT) of the

periventricular area. These latter cholinergic pathways form the main cholinergic input of thalamus.

Morphological features and connections of PPN

The PPN can be most easily identified with labelling of the cholinergic neurons. The location of cholinergic neurons is usually visualized with immunohistochemistry by using monoclonal antibody raised against the choline-acetyltransferase (ChAT) enzyme. Cholinergic cells in this area are 40% greater than choline-acetyltransferase immunonegative cells. It has been recently demonstrated that there is a gradient in the density of cholinergic neurons within PPN from rostral direction to caudal direction, i.e. the number and proportion of cholinergic cells increase towards the caudal part of PPN.

The PPN has a well known connection with several structures, such as the basal forebrain, spinal cord, pontomedullar reticular formation, cerebellum, substantia nigra, globus pallidus and the lateral part of the hypothalamus. The descending pathways from the PPN are known to terminate on the deep cerebellar nuclei, the pontomedullar reticular formation, the medioventral medulla oblongata, and a weak cholinergic innervation of the spinal cord was also identified.

The PPN and the LDT shows a significant overlap in their projections, but the LDT rather provides innervation for midline and limbic structures, whereas the PPN projects to lateral motor nuclei. Areas of the PPN and the LDT partially overlap with some other nuclei, like substantia nigra (SN) and locus coeruleus (LC). It has been shown that some of their projections run parallel to each other. There is reciprocal communication between cholinergic and cholinceptive structures.

Cell types and electrophysiological markers of the PPN

The PPN is not a homogeneous structure, as it consists of cells with different neurochemical markers. In addition to cholinergic neurons, numerous GABAergic and glutamatergic neurons can also be found, which show different density along the rostro-caudal axis of the nucleus. In the parasagittal axis of the nucleus, compared to cholinergic and glutamatergic neurons, GABAergic neurons are more densely located in the rostral part of the pedunculopontine nucleus. In contrast, glutamatergic cells are found in larger density in the caudal part of PPN. Further morphological subgroups can be distinguished within these three groups; for example, cells in PPN may differ in type and proportions of the calcium binding proteins expressed by them.

Based on their membrane properties, different functional groups of PPN neurons can be also distinguished. According to Kang and Kitai, the following three types of neurons can be distinguished by their membrane properties: type I with A current (transient outward current, which delays repolarization to the initial membrane potential after a hyperpolarizing pulse), type II which has low threshold calcium spikes and type III, whose cell membranes do not possess these features, and does not fit the previous two groups. Leonard and Llinas identified three types of neurons based on their electrophysiological markers as well. The first group of PPN neurons possessed a TTX-insensitive calcium conductance elicited by depolarization preceded by hyperpolarization, which generated burst firing pattern. Neurons in the second group had similar low threshold calcium spikes together with A-current. The third group includes neurons having A-currents but lacking low threshold calcium spikes.

The cholinergic and non-cholinergic neurons of PPN can also be grouped by their *in vivo* firing properties. In synchronized cortical activity resembling to physiological slow wave activity in anesthetized rats, PPN cholinergic cells showed two types of firing pattern. The greater population consists of slow firing cholinergic neurons, which showed activity in phase with the active 'up' state of the oscillations.

There is a small group of fast firing cholinergic neurons which were active during the 'down' state of the slow wave activity. Some non-cholinergic neurons, which are mostly putative glutamatergic neurons, can also be divided into two groups: neurons in one group are fast firing and show synchronicity with the 'down' state of cortical oscillations, the other neuronal group consists of slow firing or quiescent neurons, with a firing pattern independent from the cortical slow oscillations.

PPN produces measurable extracellular oscillations. In PPN, the presence of high amplitude alpha oscillations (8-13 Hz) is physiologically important. The amplitude of these oscillations is reduced when Parkinson's disease develops. An in vitro study has shown that almost all PPN neurons can produce gamma-frequency membrane potential oscillations when they are depolarized.

The role of PPN and its neuromodulatory regulation

The PPN, as a cholinergic nucleus of RAS, is involved in the regulation of sleep-wake cycles. It has been extensively demonstrated that PPN neurons increase their firing frequency during wakefulness and REM ("rapid eye movement") sleep, but reduce their firing frequency during slow wave sleep. Similar to cholinergic neurons, some

GABAergic and glutamatergic neurons also fire with higher frequency during wakefulness and REM sleep.

This nucleus, as one of the major cholinergic nuclei in the midbrain, does not only provide cholinergic input to many brain regions, but is itself a target for many neuromodulation mechanisms.

In vivo studies have shown that glutamate injection into PPN increased the duration of paradoxical sleep and wakefulness. Injecting NMDA promoted wakefulness in the experimental animal, whereas injection of kainate receptor agonist only increased the duration of paradoxical sleep. When the acetylcholine receptor agonist carbachol was injected to the PPN of the anesthetized animal, gamma oscillations appeared on the cortical EEG recording.

Median and dorsal raphe nuclei are connected with PPN together with providing serotonergic innervation to many other nuclei. Serotonine is able to modify the activity of PPN neurons via the receptor subtypes 5-HT₁, 5-HT₂ and 5-HT₇. Serotonine receptor stimulation enhances wakefulness while inhibiting REM sleep, and inhibits the activity of PPN REM-on (cholinergic) neurons via a postsynaptic targets.

Orexin, known to play important role in regulating food intake, also affects PPN cells as both subtypes of the orexin receptor are expressed by them. Orexin is able to inhibit both REM and non-REM sleep, and stabilizes wakefulness. Ghrelin can also affect the cholinergic neurons of PPN, by depolarizing those neurons which respond to orexin as well.

The M-current

The M-current is a voltage-dependent, low threshold, slow-activating, non-inactivating potassium current capable of regulating the excitability of neurons. It can be inhibited by the activation of a variety of metabotropic receptors, including muscarinic acetylcholine, angiotensin, substance P, purinergic, serotonin, and metabotropic glutamate receptors. The channel responsible for the M-current is formed by subunits of the KCNQ channel family (Kv7 with other nomenclature) which consist of subunits named KCNQ1-5 (Kv7.1-5). Since the activation of a number of receptors can trigger the closure of the M-channel, one or more common secondary messenger molecules mediate this process. Receptors inhibiting the M-channel are mainly G protein-linked receptors that activate the $G_{q/11}$ protein. Maintaining the responsiveness of the M-current to depolarizing stimuli, a certain PIP_2 level is required in the cell. The best known pathway is when G_q

activation triggers the hydrolysis of PIP_2 , thus inhibiting the M-current. As the inhibition of the M-current increases the excitability of the neurons, this may contribute to development of epileptic seizures.

The endocannabinoid signaling

Endocannabinoids are important regulators of neurotransmission. According to the classic description, type 1 cannabinoid (CB1) receptor is located on the neurons presynaptically and thus plays an important role in the function of the synapses. When endocannabinoids are bound to the receptor, $G_{i/o}$ protein is activated, which mediates different effects through secondary messenger molecules. Endocannabinoids are structurally membrane lipid derivatives and have been named from one of the active substances of *Cannabis sativa*, the Δ^9 -tetrahydrocannabinol (Δ^9 -THC) which is capable of binding to the same G protein coupled receptor. In the brain and on the periphery, the arachidonoil ethanolamide (AEA, also known as anandamide) and the 2-arachidonoyl glycerol (2-AG) endocannabinoids are prototypical endocannabinoids.

Cannabinoids classically activate two types of receptors, such as cannabinoid-1 (CB1) and cannabinoid-2 (CB2) receptors. In addition to

classical receptors, novel receptors (such as GPR55 and GPR119) are also known.

Endocannabinoids are stored in precursor form in the plasma membrane so they are synthesized and then released as a result of the stimulation required for their release.

Endocannabinoids have an important functional role in the retrograde regulatory mechanisms of the synapses. Cannabinoid receptors are located in the presynaptic membrane, thus endocannabinoids released from the postsynaptic neuron as a result of postsynaptic depolarization can bind to presynaptic neuron receptors and inhibit neurotransmitter release. This inhibition can be short-term depression (STD) or long-term depression (LTD), so they contribute to short-term and long-term synaptic plasticity as well. Inhibition may occur in inhibitory (depolarization-induced suppression of inhibition: DSI) and excitatory (depolarization-induced suppression of excitation: DSE) synapses.

It has been recently demonstrated that, in addition to the neurons, astrocytes have functional CB1 receptors too.

Astrocyte-neuron communication

There is growing interest towards the role of glial cells in controlling neuronal functions via various mechanisms. Glial cells have several different types. In the central nervous system, one can find macroglia, such as astrocytes, oligodendrocytes and ependymal cells; and microglia. Schwann cells and satellite cells can be found in the periphery. Within the group of astrocytes, there are further subgroups depending on their functional role or their location within the nervous system. Astrocytes play a role in forming the morphological structure of synapses and contribute to their proper functions. In addition, they mediate homeostatic effects, such as regulation of the ionic composition of the extracellular space and the level of neurotransmitters. In synapses, they form a so-called „tripartite synapse” with pre- and post-synaptic cells with active feedback connections between them. It has been shown that astrocytes have several neurotransmitter receptors, such as ionotropic and metabotropic glutamate-, CB1, GABA_B and other metabotropic neurotransmitter receptors.

Astrocytes are electrically non-excitabile, they are not capable of producing action potentials, but are characterized by intrinsic cellular excitability of intracellular changes in Ca^{2+} concentration (so-called "calcium excitability"). The emergence of Ca^{2+} waves leads to the

release of gliotransmitters (e.g. glutamate), which may affect the function of the synapses around the astrocytes in different ways.

Optogenetic activation of astrocytes

Optogenetics is a new, rapidly developing research technique that might be considered as a therapeutic option in the distant future. The essence of the technique is that light-gated ion channels or transporters found in algae or bacteria are selectively expressed by certain cell lines, providing possibility to influence excitability of the cells expressing this protein by illuminating it. The most widely used ion channel is channelrhodopsin-2 (ChR2), which was used in our study for optogenetic activation of astrocytes. This channel is originally expressed by the *Chlamydomonas reinhardtii* algae and can be activated by 470 nm blue light. ChR2 is a cation channel which is opened by blue light, permeable to Na^+ , K^+ , Ca^{2+} and H^+ ions. Activation of this cation channel depolarizes the neurons, and this leads to action potential firing. Activation of astrocytes is only partially due to depolarization, but calcium influx and intracellular acidosis (and probably by swelling of astrocytes) also contributes to development of its actions.

Neuronal currents activated by astrocytes

Astrocytes are able to influence neuronal excitability by regulating the concentration of extrasynaptic neurotransmitters. Extrasynaptic currents elicited by astrocytes have been observed in the central nervous system, which can be grouped according to magnitude, kinetics, direction, the type of neurotransmitters and gliotransmitters that trigger them, and their receptors.

Classifying the extrasynaptic currents based on their direction and duration, one can distinguish phasic outward or inward, and tonic excitatory or inhibitory currents.

The phasic slow inward currents (SICs) can be easily distinguished from the excitatory postsynaptic currents (EPSCs), based on their differences in current magnitude and kinetics. They have greater amplitude, slower rise time and decay time. It is well established that activation of extrasynaptic NMDA receptors, stimulated by both glutamate and NMDA receptor co-activator D-serine.

Slow outward currents (SOC) are less common in the central nervous system than SICs. It is more difficult to isolate from inhibitory

postsynaptic currents (IPSC). SOC_s have been shown to be triggered by the activation of GABA_A receptors.

Tonic excitatory currents have also been shown in several brain structures. Their astrocytic origin is not as exclusive as in the case of SIC_s, since neurotransmitter spillover from synapses or their volume transmission can also elicit them. The tonic excitatory currents are generated by the extracellular glutamate and the NMDA receptor co-activator glycine. The tonic inhibitory currents are generated by the activation of extrasynaptic GABA_A receptors. In addition to GABA, glycine can also generate tonic inhibitory currents.

OBJECTIVES/AIMS

In our work, we investigated the mechanisms involved in PPN neuromodulation regulation. We sought evidence whether there are neuromodulatory actions only exclusively activating neurons or there are neuromodulatory pathways involving astrocytes as well.

We were looking answers for the following questions:

1. Is there an M-type potassium current on the PPN neurons? Does the presence of M-current depend on cell types?
2. What are the effects of the M-current on the electrophysiological characteristics of neurons?
3. What is the role of astrocytes and metabotropic glutamate receptors in the tonic stimulation or inhibition of PPN neurons caused by CB1 receptor stimulation?
4. Does presynaptic CB1 receptor activation exert an inhibitory effect on the synaptic neurotransmission of PPN? Can astrocyte-dependent effects modify the actions of the presynaptic CB1 receptor activation?

MATERIALS AND METHODS

Solutions

In the present study, artificial cerebrospinal fluid (aCSF) was used. For preparation of the brain, a modified cerebrospinal fluid with low Na concentration was used, in which the NaCl was partially replaced with sucrose and glycerol.

For the electrophysiological experiments, an internal solution containing K-gluconate was used in the micropipette, the osmolarity of it was identical with the intracellular osmolarity. The internal solution also contained biocytine which was added directly into it prior to the experiment.

Calcium concentration measurement was performed using the acetoxymethyl ester (AM) form of Oregon Green 488 1,2-bis (o-aminophenoxy) ethane N, N, N', N'-tetraacetic acid (BAPTA-1) cell permeable fluorescent indicator.

Animals and preparation

9-16 day mice were employed from both sexes. For some experiments, strains expressing tdTomato fluorescent protein in GAD2- (glutamate decarboxylase 2-) or in ChAT- (choline acetyltransferase-) dependent way were used. For breeding of these mice, the cre-lox system was used where one parent expresses the promoter-specific recombinase, while the genome of the other parent contains the protein gene to be expressed, but its transcription is prevented by a stop codon bordered by two lox-P sites. For our further experiments, we used mice expressing channelrhodopsin-2 (ChR2) in glial fibrillary acidic protein –dependent way (GFAP). In some experiments, constitutional CB1 receptor knockout mice were also employed. In some cases, 9-16 days

old C3H (n = 7) and C57B16 (B6) mice (n = 5) were also used from both sexes.

The animals were decapitated and 200 μm thick coronal midbrain slices were prepared by using a vibratome. The slices were incubated at 37° C in normal aCSF for 60 minutes prior to the recording, while continuously bubbling the chamber containing the slices with carbogen.

Electrophysiology

Our experimental setup included a Zeiss Axioscope microscope equipped with a fluorescent imaging system composed of a xenon bulb based Polychrome V light source, a CCD camera, an imaging control unit (ICU) and a Till Vision software (Till Photonics). This system allows to observe changes of the fluorescence intensity in time. For whole-cell patch-clamp measurements, an Axopatch 200A amplifier was used both in voltage clamp and in current clamp mode. Data was recorded and processed using Clampex 10.0 software (Axon Instruments).

Recordings were performed at room temperature. The slices were oxygenated continuously during the recording. Patch pipettes with

5-6 M Ω resistance were fabricated from borosilicate glass capillaries using a Narishige PC-10 pipette puller. When measuring the M-current, the neurons were treated with 1 μ M tetrodotoxin and held at -20 mV, followed by 1-second repolarizing steps from -60 to -30 mV. For the pharmacological isolation of the M-current, 20 μ M XE991 was used, which is a specific M-current specific blocker. Current-clamp measurements were performed using 1 s long hyper- and depolarizing steps from -30 to +120 pA, and adaptation index (AI) was calculated from the obtained data. To measure the membrane potential oscillations, a 2 second long ramp protocol was used, which represents a linear increase in current injection with a maximum current value of 800 pA. These measurements were performed in the presence of TTX. For tonic currents, spontaneous and miniature EPSCs and IPSCs recorded in voltage-clamp mode, the holding potential was set to -60 mV. In some experiments, we electrically stimulated the neuronal presynaptic fibers innervating the investigated neuron with a monopolar electrode, which was placed 50-100 μ m away from the subject. The stimulatory electrode was connected to a BioStim STC-7a stimulator (Supertech). Optogenetic stimulation was performed on samples from GFAP-ChR2 mice, for the epifluorescence stimulation of the entire visual field (480 ± 5 nm) a xenon bulb based Polychrome V light source and Till Vision software were employed.

In some experiments, the CB1 receptor agonist WIN55,212-2 was used at a concentration of 1 μ M, while another CB1 receptor agonist ACEA was employed at 5 μ M. For some experiments, 10 μ M NBQX, 50 μ M D-AP5, 1 μ M strychnine and 10 μ M bicuculline were used to inhibit fast synaptic neurotransmission. To block metabotropic glutamate receptors, CPCCOEt, MPEP and LY341495 were used.

Intracellular calcium imaging

Prior to the measurement, slices were incubated for 45 minutes with 33 μ M Oregon Green 488 BAPTA-1 AM (OGB) or 20 μ M Rhod-2 AM for some of our experiments. Calcium signals were taken from the cell's soma filled with the calcium indicator dye. Changes of the fluorescence were recorded with the CCD camera and analyzed with the Till Vision software.

Visualization of biocytin labeled cells

Cells labelled with biocytin during recordings were visualized with using Alexa 488 fluorescent dye conjugated streptavidin. After fixation with 4% paraformaldehyde, retrieval was performed in Tris buffered saline for 60 minutes, supplemented with Triton-X and bovine serum, and incubated with the Streptavidin-Alexa 488 for 90 minutes.

Immunohistochemistry

Immunohistochemical experiments were carried out in collaboration with the Department of Anatomy, Histology and Embriology. Three wild-type B6 mice were used for these experiments. The animals were fixed with transcardial fixation, followed by removal of the brain and postfixation, and then slices were prepared. Slices were first incubated with primary antibody solutions for 2 days at 4°C followed by incubation with a mixture of appropriate secondary antibodies.

RESULTS

Only cholinergic neurons possess M-current

In the first part of our experiments, one of the direct neuromodulatory actions of PPN neurons, the M-current was investigated.

For experiments investigating the presence of the M-current in the PPN, we used mouse strains expressing the fluorescence protein tdTomato in GAD2- or ChAT- promoter-dependent way. The M-current is recorded by using the M-current relaxation protocol, and the current-specific blocking agent XE991 was used to detect the drug-sensitive current. Using the M-current relaxation protocol, negative

voltage steps from -60 to -30 mV were used from -20 mV holding potential, in order to observe the slow closure of the M-current. The average amplitude of the M-current on cholinergic neurons (recorded during the -40 mV voltage step) was -53.9 ± 4.8 pA. The M-current can also be determined by administering its selective blocker, XE991, at a concentration of 20 μ M. Determining the difference between control and XE991-resistant currents can reveal the M-current. Unlike cholinergic cells, PPN GABAergic cells did not have a current similar to the M-current (n = 36).

The M-current determines the electrophysiological differences between cholinergic and GABAergic neurons

We investigated whether the presence or absence of the M-current can cause any electrophysiological difference between cholinergic and GABAergic cells of the PPN. During current-clamp recordings, 1-second rectangular current pulses were used with 50 and 100 pA amplitudes, while the membrane potential was set manually to -60 mV. Significant frequency differences between the two cell types were observed in the action potential trains elicited by these pulses. The adaptation index (AI) is a measure of the decrease of action potential frequency within a train of action potentials. The cholinergic neurons displayed great adaptation while GABAergic cells had much lower AI

values. Medium and slow afterhyperpolarizations are considered to be influenced by the M-current. Afterhyperpolarization was always significantly greater in cholinergic cells than in GABAergic cells. In order to find out whether the differences between the cell types in question were caused by the M-current, the specific inhibitor of the M-current, XE991 was used. This caused a decrease in the amplitude of firing frequency, AI and afterhyperpolarization in cholinergic neurons. Application of XE991 did not cause any significant change in GABAergic cells. The data obtained show that the presence of the M-current plays a significant role in the differences between cholinergic and GABAergic neurons in firing frequency and its adaptation.

High-threshold membrane potential oscillations are modulated by the M-current

In the next set of experiments, high-threshold membrane potential oscillations were investigated. Based on the literature data, these membrane potential changes are TTX-resistant, with P/Q and N-type calcium channels and potassium channels are involved. A 1 second ramp current injection was used with a maximum current of 800 pA. During these experiments, we found that most cholinergic neurons had high threshold membrane potential oscillations, while in the case of GABAergic neurons none of the large amplitude oscillations were

observed. As a result of the XE991, the power of the oscillations greatly decreased.

The synaptic and extrasynaptic effects of stimulation of CB1 receptors on PPN neurons

In the second half of our experiments we have shown that, besides the direct neuronal effects, neuromodulation mechanisms have probably an astrocyte-dependent component. We have found that activation of astrocytes is able to induce metabotropic glutamate receptor (mGluR) dependent depolarization or hyperpolarization on PPN neurons.

In a previous study, our laboratory demonstrated that type 1 cannabinoid (CB1) receptor agonists elicited heterogenous neuronal reactions: some neurons depolarized, others hyperpolarized, while others did not respond. In the present work, we aimed to observe the effects of cannabinoid receptor stimulation on postsynaptic currents and tonic currents in order to separate the various cannabinoid effects. Current measurement was carried out in voltage-clamp mode at -60 mV. Using the CB1 agonist WIN55,212-2 (1 μ M) elicited tonic currents with amplitude ranging between + 22 and -22 pA (n = 17). In some cases, after application the CB1 receptor agonist, we observed slow inward currents (SIC) as well. In the CB1 knockout samples we found

only a minimal change in the holding current which had significant difference with recordings performed on wild type samples. Next, we attempted to isolate the direct neuronal effects of CB1 receptor stimulation from the indirect, astrocyte-dependent mechanisms. Slices were preincubated with 1 μ M thapsigargin to inhibit the calcium excitability of astrocytes. After treatment with thapsigargin, the spontaneous fluctuation of the holding current was significantly increased compared to the untreated samples, but the CB1 receptor agonist did not elicit further changes of the holding current ($n = 8$). In the rest of our experiments, we observed cannabinoid effects on postsynaptic currents. The holding potential was set to -60 mV when currents were measured. Both spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs) were seen during the measurement. Adding WIN55,212-2, the frequency of sEPSCs was increased and the frequency of sIPSCs was decreased, while their amplitude did not change. Preincubation with thapsigargin influenced the frequency change of sEPSCs induced by cannabinoid agonists. Instead of frequency increase of sEPSCs, we saw a significant decrease of it; but the actions on sIPSCs did not differ from those seen without thapsigargin. It can be assumed that the cannabinoid effect on synaptic events is a mixture of a direct

presynaptic inhibitory effect via a CB1 receptor and an indirect astrocytic effect enhancing excitatory neurotransmission.

Effects of optogenic activation of astrocytes on PPN neurons

In order to separate of the astrocyte dependent effects from direct neuronal CB1 receptor stimulation in a more precise way, optogenetics was used to selectively activate PPN astrocytes. First, we prepared slices from wild type and GFAP-ChR2 mice expressing the light-activated ion channel channelrhodopsin-2 (ChR2), which was used to observe the neuronal response to astrocytic stimulation. The whole visual field was illuminated and neurons were recorded in whole-cell configuration in current-clamp mode. As a result of the illumination, the neurons were depolarized and their firing frequencies increased. In voltage-clamp mode, tonic currents developed. In a few cases, hyperpolarization and the lack of response was also observed. Using mGluR inhibitors, it was also found that blocking group II mGluRs reduced the amplitude of the inward currents and the change in the holding current was significantly different from those seen under control conditions. As a result of the illumination, the amplitude and frequency of the EPSCs was also increased. Group II mGluR blockers almost completely prevented frequency increase ($n = 12$). Control experiments were also performed on GFAP-cre animals under the same

conditions as for GFAP-ChR2 animals, but the illumination did not exert any effect on these samples.

Direct synaptic effects induced by CB1 receptor stimulation

In the next experiment, we investigated the effect of the presynaptic CB1 receptor activation on the excitatory postsynaptic currents (EPSCs). To achieve this, we used paired pulse stimulations with 50 Hz frequency, and stimulated the presynaptic fibers in the nucleus. The ratio of the second and first elicited EPSCs were monitored (paired pulse ratio, PPR) during the measurement. EPSCs were continuously detected to find out whether depolarization-induced suppression of excitation (DSE) exists in PPN; while the postsynaptic neuron was depolarized. Both postsynaptic depolarization and application of WIN55,212-2 caused a similar change: a decrease in EPSC amplitude and an increase of PPR. These cannabinoid effects were missing in CB1 knockout animals ($n = 17$). The decrease of the amplitude with the increase of PPR suggests the presence of presynaptic inhibition. When stimulation of the presynaptic fibers was performed together with the optogenic activation of astrocytes, no significant change was observed on the evoked EPSCs ($n = 7$).

After investigating the evoked EPSCs, we were looking for direct and indirect endocannabinoid effects on IPSCs: we investigated

miniature IPSCs (mIPSCs) using KCl-containing internal solutions, tetrodotoxin (TTX), and ionotropic glutamate receptor blockers (D - AP5 and NBQX). The depolarization of the postsynaptic cell and the application of WIN similarly reduced the frequency, but did not affect the amplitude (n = 5).

Based on our experimental data described above, CB1 receptor activation has an indirect, astrocytic and a direct presynaptic effect. To confirm these results, the location of the CB1 receptor on astrocytes or presynaptically was detected with immunocytochemistry by our collaborative partners. CB1 receptor immunopositivity showed colocalization with GFAP, VGLUT2 and VGAT, but we almost never observed colocalization with ChAT.

DISCUSSION

The pedunculopontine nucleus - a brainstem nucleus regulating sleep and wakefulness – is under control of significant neuromodulatory mechanisms. These mechanisms are well described in certain cases, and in other cases they likely contribute to homeostatic sleep control. Based on the results above, it seems likely that neuromodulation mechanisms are only partially influenced directly by the neurons, while others develop via astrocytic activation.

The M-current is a functional marker of PPN cholinergic neurons

In the first part of my work, I was investigating a target of neuromodulatory mechanisms directly affecting neurons, the so-called M-current. In the cholinergic cells of PPN, the presence of M-current was detected, while the GABAergic cells lacked this potassium current.

Although PPN is known as the cholinergic arm of the reticular activation system, GABAergic and glutamatergic neurons also form the nucleus. PPN neurons exhibit heterogeneity in many ways, so they can be grouped according to their morphological, neurochemical properties, electrophysiological characteristic of the cells and according to their role in cortical oscillations. We have shown that cholinergic cells are likely to be identified by the presence or absence of M-current since

91% of cholinergic cells were able to produce M-current. In contrast, GAD2-positive GABAergic neurons did not show any M-current at all. Our experiments on high-threshold oscillations with more than 0.3 mV²/Hz power showed that this feature is also a functional marker of cholinergic neurons as we detected these oscillations on 82% of cholinergic neurons, while similar oscillations were not found on GABAergic cells.

The M-current has been recorded in many areas of the brain, including the brainstem. The ion channel responsible for the M-current is a slowly activating non-inactivating voltage-dependent potassium channel. The opening of the channel is controlled by several metabotropic receptors. Activation or inhibition of the M-current can change different electrophysiological properties of the neurons: due to the change in the input resistance and the resting membrane potential, the M-current can significantly control the excitability. It also determines the firing frequency of the neurons caused by depolarizing pulses and contributes to action potential firing frequency adaptation, as well as to the medium and slow afterhyperpolarization. In our experiments, we found that using identical depolarizing current step, GABAergic and cholinergic neurons fired at different frequencies. The measurements revealed that the M-current is involved in the reduction and adaptation of firing frequency and the amplitude of medium

afterhyperpolarization. Compared to the data of GABAergic cells and cholinergic cells in the presence of XE991, we found that there was no significant difference in the adaptation index at low frequency firing, but significant difference was revealed in the firing frequency elicited by higher depolarizing steps and in the amplitude of the medium afterhyperpolarization. This result indicates that the M-current of cholinergic neurons in the PPN plays an important role in firing frequency adaptation, but the presence or absence of M-current only partially contributes to the changes in firing frequency and afterhyperpolarization.

High threshold membrane potential oscillations were also recorded on several PPN neurons. According to certain literature data, this activity is in the beta-gamma frequency range while extracellularly measured in vivo human data supported the alpha-beta frequency range. Potassium-, P/Q- and N type calcium channels are responsible for this phenomenon. Our experiments showed that high-threshold oscillations are a specific feature of cholinergic neurons, which is influenced by the presence of the M-current. Using XE991, the M-current blocker, the amplitude of oscillations of cholinergic cells was significantly reduced. Our results and the short-term inhibitory effect of carbachol on oscillations known from the literature confirm the observation that

muscarinic neuromodulation has multiple targets, and the M-current constitutes only one but significant element of these.

Endocannabinoid effects have presynaptic and astrocyte dependent components as well

In the second part of our work, the neuronal presynaptic and astrocyte dependent extrasynaptic components of endocannabinoid effects on PPN neurons were separated. Cannabinoid signaling has direct presynaptic and indirect, astrocyte-dependent effects on PPN neurons. With the specific optogenetic activation of astrocytes we supported the previous results of our laboratory, that astrocytic activation causes mGluR-dependent, tonic neuronal depolarization and hyperpolarization. CB1 receptor activation has a mild stimulatory effect on the excitatory neurotransmission and inhibits the inhibitory neurotransmission. The latter is probably due to the activation of presynaptic CB1 receptors, while the effect on excitatory neurotransmission is due to two factors: presynaptic inhibition on CB1 receptors on the excitatory axon terminals and increased activity of the glutamatergic neurons due to increased release of glutamate from the astrocytes.

As a result of astrocytic activation, tonic neuronal inward and outward currents were observed. According to our laboratory's earlier

results, the depolarization elicited by CB1 receptor induced astrocytic activation can be inhibited by blocking group II mGluR receptors, while hyperpolarization can be inhibited by blocking group I mGluR receptors. It is known that astrocytes release glutamate by vesicular exocytosis and different channels and transporters, and are able to regulate extracellular glutamate levels. Glutamate released by astrocytes can affect the surrounding neurons, via their various mGluR, AMPA or NMDA receptors. It has been described in the literature that extrasynaptic mGluRs are capable of changing neuronal membrane potential and their tonic currents. We have confirmed the previous results from our laboratory about tonic depolarization and hyperpolarization, by demonstrating cannabinoid effects on tonic inward and outward currents.

The use of optogenetics allows selective activation or inhibition of certain cell populations. Astrocytes can be selectively activated by GFAP-dependent ChR2 expression and activation. Experiments with the optogenic activation of astrocytes have supported our theory that tonic changes in the excitability of PPN neurons are largely due to the astrocytic mechanisms. Optogenetic activation of astrocytes leads to stimulation of neuronal structures. The effects induced by optogenic activation and endocannabinoid effects are similar. Our data confirmed the theory that endocannabinoid effects in PPN have an indirect,

astrocytic component. Depolarization and neuronal tonic inward current are triggered by application of both CB1 receptor agonists and optogenetic activation of astrocytes and the effect is similarly prevented by blocking group II mGluR receptors.

In addition to support our preliminary data on astrocyte-dependent tonic activation and inhibition on PPN cells by several methods, we have also shown that synaptic neurotransmission is also modulated by direct and indirect endocannabinoid effects in PPN. It is well known that CB1 receptors are predominantly presynaptically located in both excitatory and inhibitory synapses. There is a growing body of evidence that, besides the presynaptic location of the CB1 receptors, astrocytes also have CB1 receptor in the various brain regions. We have also shown that both presynaptic and astrocytic CB1 receptor can be found in the PPN. Presynaptic inhibition of inhibitory and excitatory neurotransmission by the CB1 receptor is a well established phenomenon; first described in the cerebellum and the hippocampus. In the present work, we first demonstrated the presence of DSE and DSI in PPN. The CB1 receptor agonists reduced the frequency of sIPSCs and mIPSCs but did not alter their amplitude. In contrast, the frequency of sEPSCs was increased by the CB1 receptor agonist WIN55,212-2. In the case of EPSCs elicited by stimulation of presynaptic fibers, both postsynaptic depolarization and CB1 receptor

agonists caused presynaptic inhibition. It is known from the literature that increased neuronal activity, resulting in endocannabinoid production, causes astrocytic glutamate release. This glutamate is able to facilitate glutamatergic synapses and cause long-term depression via NMDA receptors. We have observed that there is an astrocyte-dependent effect increasing EPSC frequency in the PPN, that is capable of masking the inhibitory effect of presynaptic CB1 receptors. In addition, optogenic activation of astrocytes increased the frequency of sEPSCs via group II mGluR receptors. In contrast, optogenic activation of astrocytes did not affect the evoked EPSCs significantly. These observations have raised the possibility that increased excitability of glutamatergic neurons by astrocytes via group II mGluR receptors covers the presynaptic inhibition of CB1 receptor instead of a direct presynaptic, astrocyte dependent effect. The fact that inhibitory neurotransmission is not stimulated by indirect cannabinoid actions raises the possibility that there is less local GABAergic collaterals in a coronal slice preparation than glutamatergic collaterals.

SUMMARY

The pedunculopontine nucleus (PPN) is a cholinergic member of the reticular activating system. It is located in the mesopontine region, and its main physiological functions are the regulation of sleep-wakefulness cycles and movement. Several neuromodulatory actions target the PPN, being parts of homeostatic and circadian sleep regulations. Cholinergic, serotonergic, endocannabinoid, orexin- and ghrelin-mediated actions are known in the PPN. Although *in vivo* and *ex vivo* actions of these substances are documented on PPN neurons, the ways of action are not known in details. The aim of our investigation was to separate direct neuronal and indirect, astrocyte-mediated actions of cholinergic and endocannabinoid mechanisms.

We found that cholinergic neuromodulation partially influences the neurons via blockade of neuronal M-current. This low-threshold-, slowly activating potassium current was only recorded on cholinergic neurons and was not found on GABAergic or non-cholinergic ones. The M-current was proved to be responsible for spike frequency adaptation and medium afterhyperpolarization. Furthermore, it effectively modulated calcium current dependent high threshold membrane potential oscillations.

Direct neuronal and indirect, astrocyte-dependent components of endocannabinoid signaling were also investigated in the PPN. We found that the metabotropic glutamate receptor dependent tonic excitatory or inhibitory currents elicited by CB1 receptor stimulation are generated via astrocyte activation. Actions of the optogenetic astrocyte stimulation on neurons resembled to neuronal actions elicited by CB1 receptor stimulation. Besides the astrocyte-mediated tonic currents, presynaptic inhibition of excitatory and inhibitory synapses was also demonstrated. This action was only influenced by astrocytic activity via the general increase of excitability of glutamatergic neurons.



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Neptun ID: DRV36B
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List of publications related to the dissertation

1. Kovács, A., **Bordás, C.**, Bíró, T., Hegyi, Z., Antal, M., Szűcs, P., Pál, B.: Direct presynaptic and indirect astrocyte-mediated mechanisms both contribute to endocannabinoid signaling in the pedunculo pontine nucleus of mice.
Brain Struct. Funct. 222 (1), 247-266, 2017.
IF: 5.811 (2015)
2. **Bordás, C.**, Kovács, A., Pál, B.: The M-current contributes to high threshold membrane potential oscillations in a cell type-specific way in the pedunculo pontine nucleus of mice.
Front. Cell. Neurosci. 9, 2015.
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List of other publications

3. Kovács, A., **Bordás, C.**, Pál, B.: Cholinergic and endocannabinoid neuromodulatory effects overlap on neurons of the pedunculo pontine nucleus of mice.
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DOI: <http://dx.doi.org/doi:10.1097/WNR.0000000000000342>
IF: 1.343
4. Simándi, Z., Czipa, E., Horváth, A., Kőszeghy, Á., **Bordás, C.**, Pólska, S., Juhász, I., Imre, L., Szabó, G., Dezső, B., Barta, E., Sauer, S., Károlyi, K., Kovács, I., Hutóczki, G., Bognár, L., Klekner, Á., Szűcs, P., Bálint, B. L., Nagy, L.: PRMT1 and PRMT8 regulate retinoic acid-dependent neuronal differentiation with implications to neuropathology.
Stem Cells. 33 (3), 726-741, 2015.
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